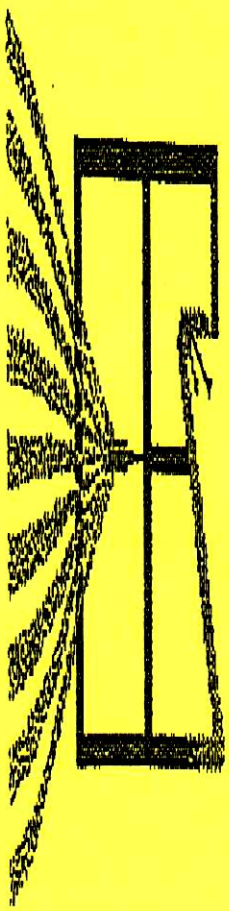
Perspectives in Percutaneous Penetration

Sixth International Conference

22-26th September 1998

Leiden, The Netherlands



Perspectives in Percutaneous Penetration

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Dear Colleague

The Organising Committee of the Sixth PPP Conference has noted your interest in the field of percutaneous penetration and would like to bring to your attention the details of the PPP 98 meeting to be held in September in Leiden. We are pleased to enclose details of the scientific programme and the registration documents.

The panel of invited speakers will review the current issues in skin permeation, whilst the workshop, topical discussions and poster sessions have been designed to maximise communication and interaction between all of the participants. In addition to the usual scientific content this particular meeting also contains a Memorial Session at which graduate students of the late Harry Boddé will make presentations on work from Leiden. We anticipate that this sixth PPP meeting will carry on the excellent tradition of previous meetings in bringing together a wide range of participants from all corners of the globe.

Local arrangements are once more being organised by Alpha Visa Congrès from Montpellier and all registration documents must be returned directly to them at the following address:

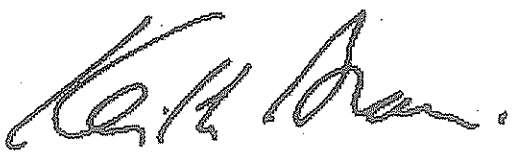
Alpha Visa Congrès / PPP Leiden 98
624 rue des Grezes
F-34070 MONTPELLIER
FRANCE

Any queries with regard to the local arrangements should be made direct to Alpha Visa Congrès / PPP Leiden 98 at the address above.

Any queries with regard to the scientific programme or abstract submission should be made to me at the address at the foot of this letter.

We look forward to meeting you in Leiden in September.

Sincerely



Keith Brain

PPP Conference, Redwood Building, Cardiff CF1 3XF,UK Tel / Fax +44 (0) 1222 874952 email : ppp@an-ex.co.uk

Consejo Superior de Investigaciones Científicas
CENTRO DE INVESTIGACION Y DESARROLLO
Jordi Girona, 18-26 · 08034 Barcelona (España)
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Enviado 29/07/98

Barcelona 29th July, 1998

PPP Conference
Redwood Building
Cardiff CF1 3XF
Wales, U.K.

Dear Sir,

Please find included the two manuscripts already accepted for presentation as posters, to be published in the Conference Proceedings. The two original camara ready manuscripts and a IBM compatible disk using Word 6.0 of Windows are submitted.

"Stratum Corneum structural modifications by effect of different solubilizing agents: a study based on high-resolution low-temperature scanning electron microscopy" O. López et al., (File LOPEZ.DOC)

"*In vivo* percutaneous absorption of liposomes and their effect on skin barrier function"
L. Coderch et al. (File CODERCH.DOC)

Please do not hesitate to contact us for any inquiry.

I remain,

Sincerely yours,

Dr. Luisa Coderch

STRATUM CORNEUM STRUCTURAL MODIFICATIONS BY EFFECT OF DIFFERENT SOLUBILIZING AGENTS: A STUDY BASED ON HIGH-RESOLUTION LOW-TEMPERATURE SCANNING ELECTRON MICROSCOPY

O. LÓPEZ[1], P. WALTHER[2], E. WEHRLI[2], M. CÓCERA[1], A. DE LA MAZA[1], L. CODERCH[1] and JL. PARRA[1]
[1]C.I.D.-C.S.I.C. Dpt. Tensioactius, Barcelona, Spain and [2]Lab. für Electron Microscopy I, ETH Zürich, Switzerland.

INTRODUCTION

A number of investigations have been devoted to improve the knowlegement about the action of different solubilizing agents on the structural organization and cohesion of the stratum corneum (SC)[1-4].

Previous studies demonstrated that whereas chloroform-methanol mixtures resulted in an almost complete removal of the intercellular lipids of the SC, the octyl glucoside surfactant (OG) showed a preferential ability to solubilize proteic material and led to a greater macroscopic disaggregation [5-6]. The different effect on the tissue cohesion of these two solubilizing agents was explained by the removal of corneocyte material and a possible partial solubilization of the corneocyte envelope by the OG. However, the difficulties to visualize the corneocyte structures and to know how these structures are affected by the solubilizing agents have to be still resolved.

A number of skin studies are based on visualization techniques; such as transmission electron microscopy applied to ruthenium tetroxide staining[7] and freeze fracture[8]. Hence, a development of this scientific field needs to be undertaken in order to avoid difficulties in the microscopical visualization and interpretation of the complex images as those of SC tissue.

Cryotechniques have now proved to be the best method to prevent drying artefacts in the study of biological materials. In this way, we have used in this work the double-layer coating for high-resolution low-temperature scanning electron microscopy (HRLTSEM) technique. This method, useful to obtain structural information comparable to that obtained with the transmission electron microscopy freeze-fracture replica, has two advantages over TEM replicas: no cleaning replica is necessary and overviews at low magnifications with high contrast are easily obtained[9].

Thus, in the present work , we seek to provide new information (using the visualization technique of HRLTSEM) about the specific effect of the organic solvent mixtures and the OG surfactant on SC tissue. To this end, we correlate the microscopic alterations in the structure of lipidic and proteic components with the macroscopic effect on the cohesion of these solubilizing agents.

EXPERIMENTAL

MATERIALS

The nonionic surfactant n-octyl β-D-glucopyranoside (OG) was purchased from Sigma Chemicals Co. (St. Louis, MO). The organic solvents chloroform and methanol were obtained from Merck (Darmstadt, Germany). Tris (hydroxymethyl)-aminonomethane (TRIS buffer) obtained from Merck was prepared as 5.0 mM TRIS buffer adjusted to pH 7.40 with HCl, contained 100 mM of NaCl.

METHODS

Isolation of stratum corneum and treatment with solubilizing agents

Sections of fresh pig skin were placed in water at 65°C for 4-5 min, and the epidermis was scraped off in sheets to isolate the SC, the epidermal sheets were incubated in 0.5% trypsin like is described in a previous paper[5].

Organic solvents: The sheets of native SC were extracted with three mixtures of chloroform-methanol (2:1, 1:1 and 1:2, v/v) at room temperature for 2 h and these extractions were reapeted for 1 h with the same solvent mixtures.

Octyl glucoside: OG buffered solutions (10 ml of 10 and 20 mM surfactant concentration) were added to the SC sheets (150 mg). The SC/OG mixture was sonicated at 25°C for about 15 min in a bath sonicator (514 ECT, Selecta) and then incubated at the same temperature for 10 h under nitrogen atmosphere[10]. This mixture was filtered to separate the SC tissue treated that was washed with abundant distilled water.

Low-temperature SEM investigation of stratum corneum samples

Cylindrical SC samples with a diameter of 2 mm and a length of about 1-2 mm were fixed with 2% (w/v) glutaraldehyde in 0.1 M sodium cacodylate buffer. The samples were cryoprotected with 30% (v/v) glycerol and mounted on a 3mm aluminium holder.

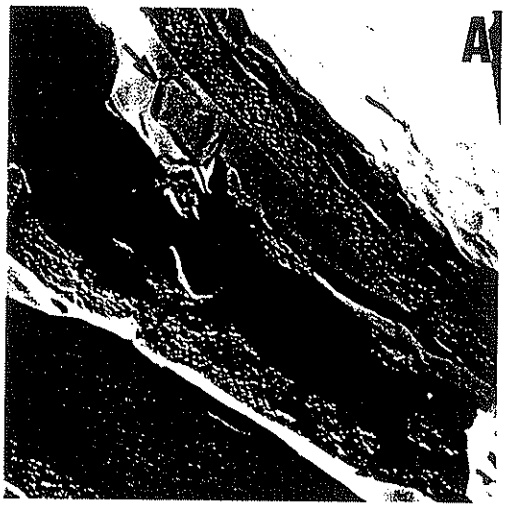
Thereafter, the SC samples were frozen by plunging into liquid propane and fractured with a microtome knife in a Balzers BAF 300 freeze-etching device (Bal-Tec., Liechtenstein) at 10^{-7}

methanol mixtures as it was confirmed by a previous study[5]. It is interesting to note that corneocyte envelope was not altered by the organic solvent extractions. In Fig. 2-A it is shown a stack of five layers of corneocytes. The smooth plane surfaces sited between these corneocytes probably corresponded to the lipids linked by covalent bonds to the amino acid residues thus forming the corneocyte envelope. We can see in this micrograph that the fracture plane occurred along the lipids of the envelope (arrow head), although some fracture across the corneocytes was also observed (arrow). A more enlarged zone where the fracture occurred across the corneocyte, is shown in Fig. 2-B. In this picture the granular structure of the corneocytes was visualized, whereas steps in the lipidic zone were not observed. From these results, the macroscopic cohesion of the SC tissue was correlated with the resistance of the corneocyte envelope and with the preservation of the structure of the corneocytes instead of the modification of the layered structure of the lipids.

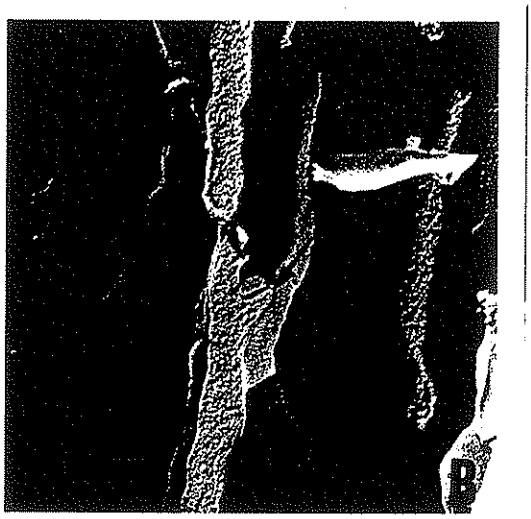
As for the sample treated with the OG, the macroscopic observation of SC showed a very different aspect to that shown by the SC extracted with organic solvents. The cohesion of the tissue was extremately affected by the surfactant which resulted in and important disagregation and in a loss of tissue cohesion (Figure 3). Thus, a serious damage in the corneocytes and in some zones a partial solubilization of the corneocyte envelope were detected (see arrow). In these zones a loss of the proteic material took place. As for the intercellular lamellar regions, lipids remained almost unaltered after the surfactant treatment, although rought structures not present in the native sample or SC extrated with organic solvents were observed in these regions (arrow heads). These rough structures could be due to the disorder in the lipid bilayers by the action of the surfactant and were associated to the loss of order detected in the X-ray diffaction patterns for samples of SC treated with OG[6]. In addition, Hofland et al[11] and Van Hal et al[12] found similar structures when they studied human SC. The mixture of the proteic material (removed from the corneocyte when the envelope was damaged) with the lipids of the intercellular spaces could be the responsible of the distorsion observed in the lipidic areas as rough structures.

Fig. 3-A show a specific zone where the aforementioned three characteristic structures in the SC treated with OG were detected. Sharp steps in the lipidic zone were observed indicating that the intercellular lipids remained in some places unaltered (open arrow). In the lipidic zones we also detected the appearance of rough structures that reveal an initial distorsion of the lipids (arrow heads). As for the corneocytes a damage in their envelopes (arrow) was also observed. This damage resulted in distorsions in the corneocyte shape (as can be observed in Fig. 3-B, arrow) before the breaking of these envelopes. The appearance of the corneocytes in these samples was always more irregular than those for the samples treated with organic solvents and SC native.

Fig. 3 Low-temperature scanning electron micrographs for the SC treated with the OG surfactant.



Magnifications — 200 nm



Magnifications — 150 nm

Given that an important loss of cohesion was observed macroscopically only in the SC treated with OG, a correlation between the microscopic alterations caused for the surfactant and the tissue cohesion seems evident. This finding is in aggrement with that previously reported[6], in which a partial solubilization of the envelope by OG was detected. The damage in the envelopes following by the loss of proteic material as well as the formation of rough structures could be responsible of the loss of cohesion in the whole of SC.

CONCLUSIONS

In the present study it has been demonstrated that HRLTSEM technique was appropriate to visualize the organization of the intercellular lipids and the corneocytes in the SC native, SC extracted with

mbar and a temperature of 168°K. After etching for 2 min, the fracture plane was coated with 2 nm platinum-carbon (unidirectional at an angle of 45° followed by 5-7 nm carbon at an angle of 90°.

Afterwards, the cold samples were immediately cryo-transferred on a Gatan cryo-holder into a Hitachi S-900, in-lents field emission scanning electron microscope equipped with a highly sensitive annular YAG-detector for back scattered electrons[9].

Speciments were investigated at 143°K. The beam current was $1\text{-}3x10^{-11}$ A as measured with a Faraday cage. The primary accelerating voltage was 10kV. Images were obtained with the back scattered electron signal and were recorded digitally with a Gatan DigiScan 688 connected to an Apple Quadra 950. Image format was 1024x1024 pixels with an integrating time of 38 μs per pixel .

RESULTS AND DISCUSSION

The use of HRLTSEM was suitable to visualize SC samples according to the method described by Walther et al[9]. This technique provided similar results to those obtained with freeze-fracture TEM replicas and offered some advantages: SC samples frozen in the native state are very brittle and the replica tend to fall into small pieces during cleaning. This problem is circunvented by cryo-SEM, because the difficult replica-cleaning process is not necessary.

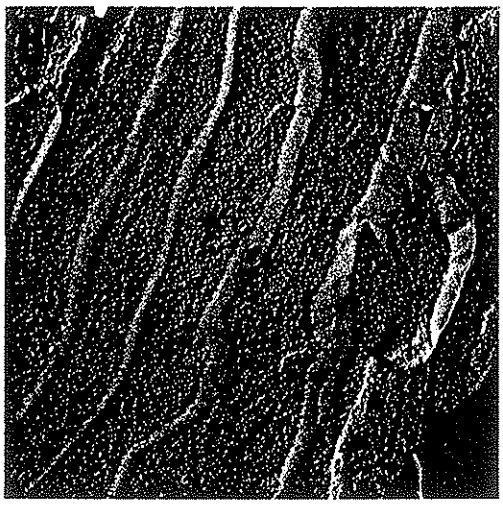
Fig. 1 Low-Temperature scanning electron micrograph of the native SC.

Magnifications — 200 nm

The micrograph obtained for native SC is shown in Figure 1. This picture depicts the corneocytes (arrow), which are characterized by the particular pattern of keratin filaments enterely filling the interior of the corneocyte and the absence of cell organelles. Mostly, the fracture plane in the corneocyte lies vertically to the skin surface. In the intercellular space, however, the fracture plane goes along the lamellae of the multilayered lipid organization. This stepwise fractured SC results in a nice view on the very smooth and relatively flat surface of the SC lipids (arrow heads). Occasionaly the bilayers were fractured straightacross, resulting in sharp edges (open arrows). This fact indicate that lipid matrix in SC is compossed of multiple layers.

Fig. 2 Low-temperature scanning electron micrographs corresponding to the SC treated with organic solvents at different magnifications

Magnifications — 960 nm



Magnifications — 200 nm

Visual inspection of SC tissue after extraction with organics solvents showed that the mixtures used were not able to promote disagregation of the tissue. Microscopic observation by SEM of the tissue is shown in Figures 2-A and 2-B. These pictures reveal that the granular appearance of the fracture plane corresponding to the corneocytes (keratin tonofilaments, arrow) remain unaltered when in SC is extracted with organic solvents mixtures. As for lipidic zones, only one layer of lipids could be observed between the corneocytes (arrow heads). This is indicative that an important part of the lipids were extracted by the chloroform-

organic solvents and treated with OG. The organic mixtures removed almost completely the intercellular lipids, however the corneocytes and their envelopes remained unaltered after extraction. The effect of the nonionic surfactant OG was different. A clear damage in the corneocyte envelope and a loss of the proteic material from the corneocyte was visualized, in addition to the formation of rough structures. The microscopic differences in the structure caused by these two solubilizing agents allowed us to explain the different macroscopic effect of these agents on the cohesion of the SC, and the different function of the SC components. Thus, the disagregation of the SC tissue takes place by breaking of the envelopes and the loss of material from the corneocyte to the lipidic zone.

ACKNOWLEDGEMENTS

We thank Mr. Luis Peña for his skillful work at the obtention of high quality images and we are also grateful to Mr. G. Von Knorring for his expert technical assistance. This work was supported by funds from DGCYT (Dirección General de Investigación Científica y Técnica) (Prog. n° PB94-0043), Spain.

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PPP 1998 Leiden (Holanda)

INTRODUCTION

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Organic solvents: The sheets of native SC were extracted with three mixtures of chloroform-methanol (2:1, 1:1 and 1:2, v/v) at room temperature for 2 h and these extractions were repeated for 1 h with the same solvent mixtures.
Octyl glucoside: OG buffered solutions (10 and 20 mM) were added to the SC sheets. The SC/OG mixture was sonicated at 25°C for about 15 min in a bath sonicator and then incubated at the same temperature for 10 h under nitrogen atmosphere[6]. This mixture was filtered to separate the SC tissue treated that was washed with abundant distilled water.

Low-temperature SEM investigation of stratum corneum samples

Cylindrical SC samples with a diameter of 2 mm and a length of about 1-2 mm were fixed with 2% (w/v) glutaraldehyde in 0.1 M sodium cacodylate buffer. The samples were cryoprotected with 30% (v/v) glycerol and mounted on a 3 mm aluminium holder. Thereafter, the SC samples were frozen by plunging into liquid propane and fractured with a microtome knife in a Balzers BAF 300 freeze-etching device (Bal-Tec., Liechtenstein) at 10^{-7} mbar and a temperature of 168°K. After etching for 2 min, the fracture plane was coated with 2 nm platinum-carbon at an angle of 45° followed by 5-7 nm carbon at an angle of 90°. Afterwards, the cold samples were immediately cryo-transferred on a Gatan cryo-holder into a Hitachi S-900, in-lens field emission SEM equipped with a highly sensitive annular YAG-detector for back scattered electrons[4]. Specimens were investigated at 143°K. The beam current was $1\text{-}3\times10^{-11}$ A as measured with a Faraday cage. The primary accelerating voltage was 10kV. Images were obtained with the back scattered electron signal and were recorded digitally with a Gatan DigiScan 688 connected to an Apple Quadra 950. Image format was 1024x1024 pixels with an integrating time of 38 µs per pixel .

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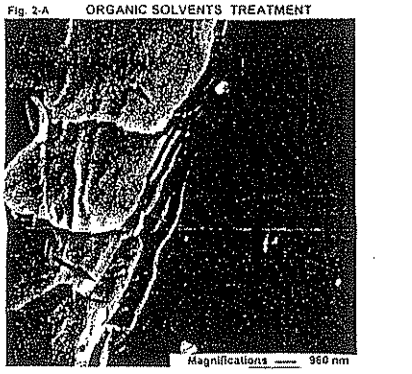
RESULTS AND DISCUSSION

Fig. 1 NATIVE SC

Magnifications —— 200 nm

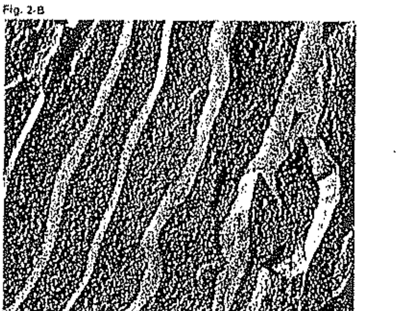
The micrograph obtained for native SC is shown in Figure 1. This picture depicts the corneocytes (arrow), which are characterized by the particular pattern of keratin filaments enterely filling the interior of the corneocyte and the absence of cell organelles. Mostly, the fracture plane in the corneocyte lies vertically to the skin surface. In the intercellular space, however, the fracture plane goes along the lamellae of the multilayered lipid organization. This stepwise fractured SC results in a nice view on the very smooth and relatively flat surface of the SC lipids (arrow heads). Occasionaly the bilayers were fractured straightacross, resulting in sharp edges (open arrows). This fact indicate that lipid matrix in SC is composed of multiple layers.

Fig. 2-A ORGANIC SOLVENTS TREATMENT



Magnifications —— 960 nm

Fig. 2-B



Magnifications —— 200 nm

Organic solvents treatment

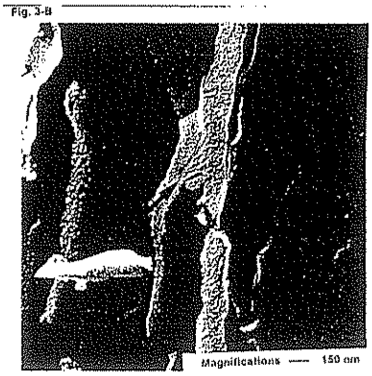
The sheets of native SC were extracted with three mixtures of chloroform-methanol (2:1, 1:1 and 1:2, v/v) at room temperature for 2 h and these extractions were repealed for 1 h with the same solvent mixtures.

Visual inspection of SC tissue after extraction with organic solvents showed that the mixtures used were not able to promote disaggregation of the tissue. Microscopic observation by SEM of the tissue is shown in Figures 2-A and 2-B. These pictures reveal that the granular appearance of the fracture plane corresponding to the corneocytes (keratin tonofilaments, arrow) remain unaltered when in SC is extracted with organic solvents mixtures. As for lipidic zones, only one layer of lipids could be observed between the corneocytes (arrowheads). This is indicative that the chloroform-methanol mixtures extracted an important part of the lipids as it was confirmed by a previous study[5]. It is interesting to note that corneocyte envelope was not altered by the organic solvent extractions. In Fig. 2-A it is shown a stack of five layers of corneocytes. The smooth plane surfaces sited between these corneocytes probably corresponded to the lipids linked by covalent bonds to the amino acid residues thus forming the corneocyte envelope. We can see in this micrograph that the fracture plane occurred along the lipids of the envelope (arrow heads), although some fracture across the corneocytes was also observed (arrow). A more enlarged zone where the fracture occurred across the corneocyte, is shown in Fig. 2-B. In this picture the granular structure of the corneocytes was visualized, whereas steps in the lipidic zone were not observed. From these results, the macroscopic cohesion of the SC tissue was correlated with the resistance of the corneocyte envelope and with the preservation of the structure of the corneocytes instead of the modification of the layered structure of the lipids.

Fig. 3-A OG TREATMENT

Magnifications —— 200 nm

Fig. 3-B



Magnifications —— 150 nm

Octyl glucoside treatment

OG buffered solutions (10 and 20 mM) were added to the SC sheets. The SC/OG mixture was sonicated at 25°C for about 15 min in a bath sonicator and then incubated at the same temperature for 10 h under nitrogen atmosphere[6]. This mixture was filtered to separate the SC tissue treated that was washed with abundant distilled water.

As for the sample treated with the OG, the macroscopic observation of SC showed a very different aspect to that shown by the SC extracted with organic solvents. The cohesion of the tissue was extremely affected by the surfactant, which resulted in and important disaggregation, and in a loss of tissue cohesion (Figure 3). Thus, a serious damage in the corneocytes and in some zones a partial solubilization of the corneocyte envelope were detected (see arrow). In these zones a loss of the proteic material took place. As for the intercellular lamellar regions, lipids remained almost unaltered after the surfactant treatment, although rough structures not present in the native sample or SC extructed with organic solvents were observed in these regions (arrow heads). These rough structures could be due to the disorder in the lipid bilayers by the action of the surfactant and were associated to the loss of order detected in the X-ray diffraction patterns for samples of SC treated with OG[6]. In addition, Hofland et al[7] and Van Hal et al[8] found similar structures when they studied human SC. The mixture of the proteic material (removed from the corneocyte when the envelope was damaged) with the lipids of the intercellular spaces could be the responsible of the distorsion observed in the lipidic areas as rough structures. In Fig. 3-A it is shown a specific zone where the aforementioned three characteristic structures in the SC treated with OG were detected. Sharp steps in the lipidic zone were observed indicating that the intercellular lipids remained in some places unaltered (open arrow). In the lipidic zones we also detected the appearance of rough structures that reveal an initial distorsion of the lipids (arrowheads). As for the corneocytes a damage in their envelopes (arrow) was also observed. This damage resulted in distorsions in the corneocyte shape (as can be observed in Fig. 3-B, arrow) before the breaking of these envelopes. The appearance of the corneocytes in these samples was always more irregular than those for the samples treated with organic solvents and SC native.

CONCLUSIONS

HRLTSEM technique was appropriate to visualize the organization of the intercellular lipids and the corneocytes in the SC native, SC extracted with organic solvents and treated with OG. The organic mixtures removed the intercellular lipids, however the corneocytes and their envelopes remained unaltered after extraction. The effect of the nonionic surfactant OG was different. A clear damage in the corneocyte envelope and a loss of the proteic material from the corneocyte was visualized, in addition to the formation of rough structures. The microscopic differences in the structure caused by these two solubilizing agents allowed us to explain the different macroscopic effect of these agents on the cohesion of the SC, and the different function of the SC components. Thus, the disaggregation of the SC tissue takes place by breaking of the envelopes and the loss of material from the corneocyte to the lipidic zone.